J. Parasitol., 88(4), 2002, pp. 790–793
© American Society of Parasitologists 2002

EXAMINATION OF ATTACHMENT AND SURVIVAL OF *TOXOPLASMA GONDII* OOCYSTS ON RASPBERRIES AND BLUEBERRIES

Kalmia E. Kniel, David S. Lindsay*, Susan S. Sumner†, Cameron R. Hackney‡, Merle D. Pierson†, and J. P. Dubey§

Department of Biomedical Sciences and Pathobiology, Virginia–Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, 1410 Prices Fork Road, Blacksburg, Virginia 24061. *e-mail: lindsayd@vt.edu*

ABSTRACT: The consumption of *Toxoplasma gondii* oocysts on fresh produce may be a means of its transmission to humans. Cats shed *T. gondii* oocysts, which contaminate produce directly or contaminate water sources for agricultural irrigation and pesticide and fertilizer applications. *Cyclospora cayetanensis* is a related coccidial parasite, and outbreaks of diarrhea caused by *C. cayetanensis* have been associated with the ingestion of contaminated raspberries. The oocysts of these coccidians are similar in size and shape, indicating that they may attach to and be retained on produce in a similar manner. In the present study the attachment and survival of *T. gondii* oocysts on 2 structurally different types of berries were examined. Raspberries and blueberries were inoculated individually with 1.0×10^1 to 2.0×10^4 oocysts of sporulated *T. gondii*. Berries inoculated with 2.0×10^4 oocysts were stored at 4 C for up to 8 wk. Oocyst viability and recovery were analyzed by feeding processed material to mice. Mice fed *T. gondii*–inoculated berries stored at 4 C for 8 wk developed acute infections. In other experiments mice fed raspberries inoculated with $\geq 1.0 \times 10^1$ oocysts became infected, whereas only mice fed blueberries inoculated with $\geq 1.0 \times 10^3$ oocysts became infected. This study demonstrates that *T. gondii* oocysts can adhere to berries and can be recovered by bioassays in mice and that raspberries retain more inoculated oocysts than do blueberries. The results suggest that *T. gondii* may serve as a model for *C. cayetanensis* in food safety studies.

Toxoplasma gondii infects a wide range of warm-blooded animals and can cause severe infection in healthy individuals and potentially life-threatening infection in newborns and in immunocompromised persons, in particular in AIDS patients as a result of reactivation of the infection (Wanke et al., 1987; Choi et al., 1997). Domestic cats and other felines are the only known definitive hosts of *T. gondii*, whereas humans are the common intermediate hosts. Cats can excrete millions of oocysts in their feces. It has been documented that the sporulated *T. gondii* oocysts can survive in the environment for 1.5 yr (Frenkel et al., 1975) and for 4.5 yr at 4 C (Dubey, 1998). Unsporulated oocysts can survive for 11 wk at 4 C and resume development when the temperature is raised to 22–25 C (Lindsay et al., 2002). This means that the oocysts have the potential to remain viable in irrigation water or in field soil for many years. Ingestion of unwashed raw fruits or vegetables has been associated with an increased risk of maternally acquired toxoplasmosis (Kapperud et al., 1996). *Toxoplasma gondii* oocysts have been isolated from soil obtained from gardens (Coutinho et al., 1982), and direct contact with soil has been associated with an increased risk of maternal toxoplasmosis (Decavalas et al., 1990). Humans become infected when they ingest food, water, or other consumable products that have been contaminated with oocysts (Sulzer et al., 1986; Isaac-Renton et al., 1997; Aramini et al., 1999).

Cyclospora cayetanensis is a related coccidial parasite, and outbreaks of diarrhea caused by *C. cayetanensis* have been associated with the ingestion of contaminated raspberries (Centers for Disease Control and Prevention, 1996, 1997, 1998; Bern et al., 1999; Sterling and Ortega, 1999). No laboratory model exists for *C. cayetanensis*, and oocysts must come from infected humans (Eberhard et al., 2000). Dubey et al. (1998) examined the effects of gamma irradiation on *T. gondii* oocysts and indicated that it might be a model for *C. cayetanensis*. The present study was done to determine the numbers of sporulated oocysts needed to attach to raspberries and how long oocysts would remain viable on the surface of raspberries. Raspberries were also compared with blueberries to determine if the surface differences in the berries would influence attachment and survival.

Received 9 January 2002; revised 12 February 2002; accepted 18 February 2002.

* To whom correspondence should be addressed.
† Department of Food Science and Technology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061.
‡ Davis College of Agriculture, Forestry and Consumer Sciences, West Virginia University, Morgantown, West Virginia 26506.
§ Parasite Biology, Epidemiology and Systematics Laboratory, ANRI, ARS, United States Department of Agriculture, Beltsville, Maryland 20705.

MATERIALS AND METHODS

Oocysts of the VEG strain (Dubey et al., 1996) of *T. gondii* were recovered from the feces of the experimentally infected cats (Dubey, 1995) housed at the United States Department of Agriculture Laboratory, Beltsville, Maryland. For sporulation, oocysts were incubated in 2% sulfuric acid for 1 wk. Sporulated oocysts were sent to the Department of Biomedical Sciences and Pathobiology, Center for Molecular Medicine and Infectious Diseases, Virginia–Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, Virginia, for subsequent testing on raspberries and blueberries and for mouse-feeding studies. The acid solution was removed from the oocysts by centrifugation in Hanks' balanced salt solution (HBSS, Fisher Scientific, Pittsburgh, Pennsylvania). Sporulated *T. gondii* oocysts were counted on a hemocytometer. Oocyst dilutions were prepared in HBSS. Two different batches of oocysts were used.

Raspberries and blueberries were purchased from a local grocery. Visibly clean berries were chosen for use in experiments and weighed but not washed before use. The average raspberry weight was 2.20 ± 0.4 g, and the average blueberry weight was 1.34 ± 0.3 g.

Spot inoculation

The berries were gently placed individually in a beaker, and a known number of oocysts (first batch of oocysts; 2.0×10^4 or fewer oocysts) in 1 ml were added dropwise onto the berry using a pipette. The berry was gently rolled in the beaker for 2 min to coat with oocysts. The berry was gently removed from the beaker, placed on a paper towel in a laminar flow hood to dry for 2 min, and then carefully placed in a whirl-pack bag (Fisher) and stored at 4 C until use. The beaker was washed thoroughly and microwaved between samples. Control berries were exposed only to HBSS.

Immersion inoculation

The berries were gently placed individually in 50-ml conical tubes containing a known number of oocysts (first batch of oocysts; 2.0×10^4 or fewer oocysts) in a total volume of 5 ml HBSS. The conical tube

was gently agitated for 2 min to distribute the oocysts over the berry surface. The berry was gently removed from the conical tube with tweezers, placed on a paper towel in a laminar flow hood to dry for 2 min, and then carefully placed in a whirl-pack bag and stored at 4 C until use. A new, sterile conical tube was used for each sample. Control berries were exposed only to HBSS.

Dose titration of oocyst infectivity

This was done to compare the numbers of infective oocysts (first batch of oocysts) that were attached to the raspberries or blueberries. In this experiment individual berries were spot inoculated with HBSS containing 2.0×10^4, 1.0×10^4, 1.0×10^3, 1.0×10^2, or 1.0×10 sporulated oocysts. The berries were placed on a paper towel in a laminar flow hood to dry for 2 min and then placed in a refrigerator for 2 hr. The berries were processed as described subsequently, and the infectivity of *T. gondii* oocysts on exposed berries was evaluated by oral inoculation of groups of 3 CD-1 mice.

Long-term survival of *Toxoplasma gondii* oocysts on berries at 4 C

The raspberries and blueberries were individually exposed to 2.0×10^4 oocysts by spot inoculation (second batch of oocysts). Three berries of each type were pooled and examined at the selected time intervals. Control berries were exposed only to HBSS. *Toxoplasma gondii* infectivity was assayed by oral feeding of processed material to groups of 3 female A/J mice after 2, 4, 6, and 8 wk of storage at 4 C.

Sample preparation and mouse inoculations

Three berries of each type were pooled in a stomacher bag containing 15 ml HBSS and stomached for 30 sec at normal speed (Seward Lab Blender Stomacher 80). The berry suspension was filtered through cheesecloth to separate oocysts from berry mash and seeds. The filtrate was concentrated at 1,500 *g* for 10 min, and the final volume of HBSS was brought to 3 ml. Oocysts were observed in the filtrate using light microscopy. The filtrate was then fed using an animal-feeding needle to a group of 3 female CD-1 mice (Experiment 1, first batch of oocysts). The berries inoculated in both ways were fed to mice 0, 2, and 6 days postinoculation (PI). Berry suspension was fed to mice within 1 hr of processing in all the experiments reported in the present study.

Examination of mice for *Toxoplasma gondii*

Mouse tissues were evaluated by direct examination of impression smears from the liver, lungs, or ascites for tachyzoites upon death. Blood was collected from the retro-orbital plexus of surviving mice 4–6 wk PI, and sera were used in the modified direct agglutination test for *T. gondii* at a 1:50 dilution (Dubey and Desmonts, 1987). Surviving mice were killed 6 wk PI and their brains removed, and fresh smears were examined for *T. gondii* tissue cysts.

Scanning electron microscopy

Scanning electron microscopy (SEM) was performed to evaluate berry topography and its role in adherence. The raspberries and blueberries were individually spot inoculated with 2.0×10^4 oocysts, fixed in 2.5% gluteraldehyde in 0.1 M sodium phosphate buffer, and coated with gold particles for SEM. The berries were examined in a JOEL 35-C scanning electron microscope.

RESULTS

Method of berry inoculation

The berries appeared visibly normal during this part of the study. None of the control mice fed berries became infected. All the inoculated mice in both treatment groups (spot and immersion inoculations) became infected with *T. gondii*. We determined that the spot inoculation method of infecting berries was easier to conduct and used it for subsequent studies reported herein.

TABLE I. Dose titration of *Toxoplasma gondii* oocysts spot inoculated on raspberries and blueberries.

Spot inoculation (no. of oocysts)	No. of mice fed*	No. of mice positive (acute)†
Raspberries		
Control	2	0 (0)
2.0×10^4 oocysts	3	3 (3)
1.0×10^4 oocysts	3	3 (2)
1.0×10^3 oocysts	3	3 (2)
1.0×10^2 oocysts	3	2 (0)
1.0×10^1 oocysts	3	1 (0)
Blueberries		
Control	2	0 (0)
2.0×10^4 oocysts	3	3 (3)
1.0×10^4 oocysts	3	2 (0)
1.0×10^3 oocysts	3	0 (0)

* Number of mice fed processed material.
† Number of mice positive for *T. gondii* (number died or killed due to acute toxoplasmosis).

Dose titration of oocysts on berries

The berries appeared visibly normal during this part of the study. None of the control mice fed berries became infected. Mice fed raspberries inoculated with $\geq 1.0 \times 10^3$ oocysts were all seropositive, and all but 2 died of acute toxoplasmosis (Table I). The raspberries inoculated with 1.0×10^2 or 1.0×10 oocysts were still infectious to mice. Mice fed blueberries inoculated with $\geq 1.0 \times 10^4$ oocysts were infected but not those fed blueberries inoculated with 1.0×10^3 oocysts (Table I).

Long-term survival of *Toxoplasma gondii* oocysts on berries

The berries developed surface mold that would make them appear unpalatable and preclude ingestion by humans after 4 wk of storage at 4 C. This mold growth continued until 8 wk PI, when the study was terminated. No attempt was made to identify the species of mold present. None of the control mice fed berries became infected. All mice fed inoculated berries after 2, 4, 6, and 8 wk of storage at 4 C became infected and died or were killed because of acute toxoplasmosis.

Scanning electron microscopy

The raspberries were covered with many tiny hairlike projections and fewer thick hairlike projections that were available to interact with *T. gondii* oocysts (Fig. 1A, B). One suspect *T. gondii* oocystlike structure associated with the fine hairlike projections was observed (Fig. 1B). The blueberries lacked hairlike structures but had spaces, crevices, and open stomata where oocysts could adhere (Fig. 1C). No *T. gondii* oocyst or oocystlike structure was observed on the surface of the blueberries examined.

DISCUSSION

The present study demonstrates that the sporulated oocysts of *T. gondii* can attach to and remain infectious on raspberries and blueberries for at least 8 wk under refrigerator conditions.

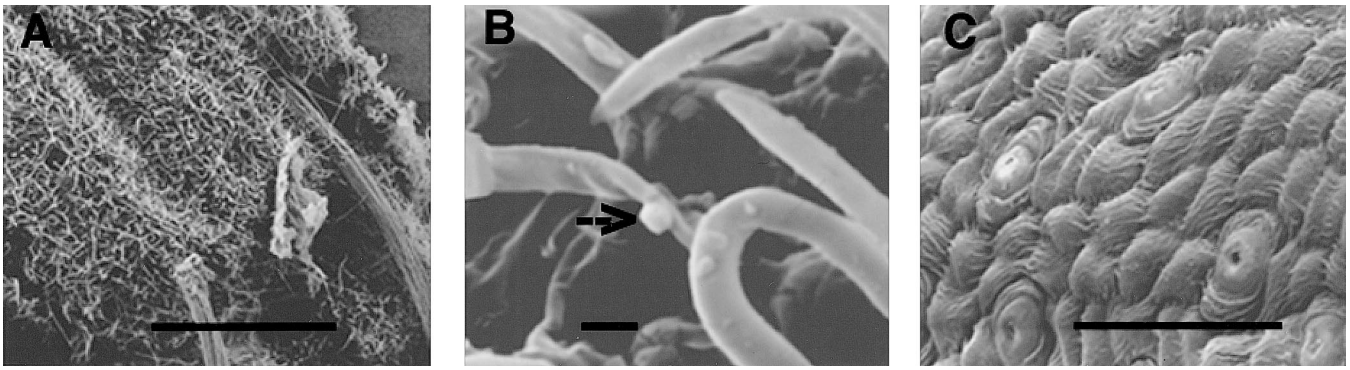

FIGURE 1. Scanning electron micrographs of the surface structure of raspberry and blueberry after spot inoculation with 2.0×10^4 *Toxoplasma gondii* oocysts. (**A**) Side surface of a raspberry showing numerous fine and few thick hairlike projections. Bar = 1 mm. (**B**) Oocystlike structure (arrow) appears to be attached to the fine hairlike structures on the raspberry surface. Bar = 10 μm. (**C**) Side surface view of a blueberry showing open stomata and crevices. Bar = 0.1 mm.

Oocysts adhered to berries by both spot and immersion inoculation methods. The spot inoculation method was chosen for subsequent studies because it is more similar to how oocysts might adhere to berries in a field setting where contamination could occur from water used for irrigation and pesticide and fertilizer applications. The presence of mold on the berries indicates that they were beginning to decay. The present study indicates that the oocysts also survive under conditions of mold growth on berries.

The dose titration studies reported herein indicate that more oocysts attach to raspberries than to blueberries. The presence of the many fine hairlike projections on raspberries (Fig. 1) probably aids in the adherence of *T. gondii* oocysts to this fruit. Entrapment in the surface covering has also been suggested as a possible attachment mechanism for *C. cayetanensis* oocysts to raspberries (Sterling and Ortega, 1999). The relatively smooth surface of the blueberries probably provides less area where oocysts can become trapped and remain attached.

As few as 1 oocyst of the VEG strain is potentially infectious for mice and pigs (Dubey et al., 1996). In the present study oocysts may have been lost during processing and filtration before mouse-feeding studies and, therefore, the numbers provided may underestimate the true numbers needed to attach and cause infection. It is likely that the numbers of oocysts evaluated in this study are present in the nonpotable water that may be used for irrigation and pesticide and fertilizer applications. Although cats can shed many thousands of *T. gondii* oocysts each day into irrigation water supplies, only a small proportion of oocysts may find their way to the field and onto plants, where they may adhere to berries as indicated in this study.

Lee and Lee (2001) recently proposed using the chicken coccidium *Eimeria acervulina* as a model coccidial organism for food safety studies. They used *E. acervulina* oocysts to infect raspberries and determined the effects of various treatments for removal and inactivation of *E. acervulina* oocysts. They believed that *E. acervulina* might serve as a potential surrogate for *C. cayetanensis*. However, *T. gondii* oocysts may serve as a better surrogate and model for *C. cayetanensis* because they are more similar in size and shape. Both oocysts are spherical to slightly subspherical, with *T. gondii* measuring 12×10 μm and *C. cayetanensis* measuring 10×8 μm. Additionally, both coccidians have 2 sporocysts in their oocysts. The sporocysts are also similar in size, with the *T. gondii* sporocyst measuring 8×6 μm and the *C. cayetanensis* sporocyst measuring 6×4 μm. Both *T. gondii* and *C. cayetanensis* oocysts infect humans. The present study adds to the development of *T. gondii* oocysts as a model for *C. cayetanensis* oocysts on raspberries and other produce. The results can be used by those interested in examining ways to remove viable coccidial oocysts from the produce.

ACKNOWLEDGMENTS

This work was supported in part by a New Initiative Grant from the Virginia–Maryland Regional College of Veterinary Medicine, CSREES USDA Special Food Safety Grant 98-34382-6916, and VPI & SU HATCH project 135563. We thank Alexa C. Rosypal, Kay Carlson, and Sheila M. Mitchell, Department of Biomedical Sciences and Pathobiology, Virginia–Maryland Regional College of Veterinary Medicine, Blacksburg, Virginia, for technical assistance.

LITERATURE CITED

ARAMINI, J. J., C. STEPHEN, J. P. DUBEY, C. ENGELSTOFT, H. SCHWANTJE, AND C. S. RIBBLE. 1999. Potential contamination of drinking water with *Toxoplasma gondii* oocysts. Epidemiology and Infection **122:** 305–315.

BERN, C., B. HERNANDEZ, M. B. LOPEZ, M. J. ARROWOOD, M. A. DE MEJIA, A. M. DE MERIDA, A. W. HIGHTOWER, L. VENCZEL, B. L. HERWALDT, AND R. E. KLEIN. 1999. Epidemiologic studies of *Cyclospora cayetanensis* in Guatemala. Emerging Infectious Diseases **5:** 774–776.

CENTERS FOR DISEASE CONTROL AND PREVENTION. 1996. Outbreaks of *Cyclospora cayetanensis* infection—United States, 1996. Morbidity and Mortality Weekly Reports **45:** 549–551.

CENTERS FOR DISEASE CONTROL AND PREVENTION. 1997. Update: Outbreaks of cyclospoiasis—United States and Canada, 1997. Morbidity and Mortality Weekly Reports **46:** 521–523.

CENTERS FOR DISEASE CONTROL AND PREVENTION. 1998. Outbreak of cyclosporiasis—Ontario, Canada, May 1998. Morbidity and Mortality Weekly Reports **47:** 806–809.

CHOI, W. Y., H. W. NAM, AND N. H. KWAK. 1997. Foodborne outbreaks of human toxoplasmosis. Journal of Infectious Diseases **175:** 1280–1282.

COUTINHO, S. G., R. LOBO, AND G. DUTRA. 1982. Isolation of *Toxoplasma* from the soil during an outbreak of toxoplasmosis in a rural area in Brazil. Journal of Parasitology **68:** 866–868.

DECAVALAS, G., M. PAPAPETROPOULOU, E. GIANNOULAKI, V. TZIGOUNIS, AND X. G. KONDAKIS. 1990. Prevalence of *Toxoplasma gondii* antibodies in gravidas and recently aborted women and study of risk factors. European Journal of Epidemiology **6:** 223–226.

DUBEY, J. P. 1995. Duration of immunity to shedding of *Toxoplasma gondii* oocysts by cats. Journal of Parasitology **81:** 410–415.

———. 1998. *Toxoplasma gondii* oocyst survival under defined temperatures. Journal of Parasitology **84:** 862–865.

———, AND G. DESMONTS. 1987. Serological responses of equids fed *Toxoplasma gondii* oocysts. Equine Veterinary Journal **19:** 337–339.

———, J. K. LUNNEY, S. K. SHEN, O. C. H. KWOK, D. A. ASHFORD, AND P. THULLIEZ. 1996. Infectivity of low numbers of *Toxoplasma gondii* oocysts for pigs. Journal of Parasitology **82:** 438–443.

———, D. W. THAYER, C. A. SPEER, AND S. K. SHEN. 1998. Effect of gamma irradiation on unsporulated and sporulated *Toxoplasma gondii* oocysts. International Journal for Parasitology **28:** 369–375.

EBERHARD, M. L., Y. R. ORTEGA, D. E. HANES, E. K. NACE, R. Q. DO, M. G. ROBL, K. Y. WON, C. GAVIDIA, N. L. SASS, K. MANSFIELD, A. GOZALO, J. GRIFFITHS, R. GILMAN, C. R. STERLING, AND M. J. ARROWOOD. 2000. Attempts to establish experimental *Cyclospora cayetanensis* infection in laboratory animals. Journal of Parasitology **86:** 577–582.

FRENKEL, J. K., A. RUIZ, AND M. CHINCHILLA. 1975. Soil survival of *Toxoplasma* oocysts in Kansas and Costa Rica. American Journal of Tropical Medicine and Hygiene **24:** 439–443.

ISAAC-RENTON, J., W. R. BOWIE, A. KING, G. S. IRWIN, C. S. ONG, C. P. FUNG, M. O. SHOKEIR, AND J. P. DUBEY. 1997. Detection of *Toxoplasma gondii* oocysts in drinking water. Applied Environmental Microbiology **64:** 2278–2280.

KAPPERUD, G., P. A. JENUM, B. STRAY-PEDERSEN, K. K. MELBY, A. ESKILD, AND J. ENG. 1996. Risk factors for *Toxoplasma gondii* infection in pregnancy. Results of a prospective case–control study in Norway. American Journal of Epidemiology **144:** 405–412.

LEE, M. B., AND E. H. LEE. 2001. Coccidial contamination of raspberries: Mock contamination with *Eimeria acervulina* as a model for decontamination treatment studies. Journal of Food Protection **64:** 1854–1857.

LINDSAY, D. S., B. L. BLAGBURN, AND J. P. DUBEY. 2002. Survival of nonsporulated *Toxoplasma gondii* oocysts under refrigerator conditions. Veterinary Parasitology **103:** 309–313.

STERLING, C. R., AND Y. R. ORTEGA. 1999. *Cyclospora*: An enigma worth unraveling. Emerging Infectious Diseases **5:** 48–53.

SULZER, A. J., E. L. FRANCO, E. TAKAFUJI, M. BENENSON, K. W. WALLS, AND R. L. GREENUP. 1986. An oocyst-transmitted outbreak of toxoplasmosis: Patterns of immunoglobulin G and M over one year. American Journal of Tropical Medicine and Hygiene **35:** 290–296.

WANKE, C., C. U. TUAZON, A. KOVACS, T. DINA, D. O. DAVIS, N. BARTON, D. KATZ, M. LUNDE, C. LEVY, F. K. CONLEY, H. C. LANE, A. S. FAUCI, AND H. MASUR. 1987. Toxoplasma encephalitis in patients with acquired immune deficiency syndrome: Diagnostic and response to therapy. American Journal of Tropical Medicine and Hygiene **36:** 509–516.